Label-Free Phenotype MicroArrays™ Analysis of Cellular Energetics and Apoptotic Activity using Microplate Reading and Phase Contrast Imaging

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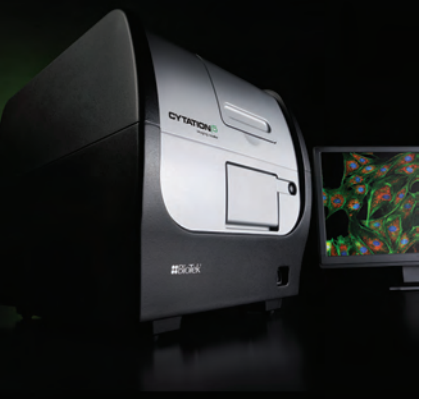

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Introduction

Phenotypic screening, or the determination of the effects (phenotypes) that a molecule has on a cell, tissue, or whole organism, dates back to earliest drug discovery efforts. Due to advances in molecular biology and biochemistry in the 1990s, this approach was de-emphasized in favor of a more "reductionist" target-based approach[1]. Mounting evidence, however, indicates this shift not only failed to accelerate discovery of new first-in-class medicines, but has also led to higher attrition rates of new lead molecules[2]. Thus, a more balanced "holistic" approach, which incorporates both discovery methods, is increasingly being emphasized.

Phenotype MicroArrays™ is a unique label-free multiplexing technology for screening cell-based energetics that describe a variety of metabolically related phenotypes in a target cell model. By varying the available cellular nutrition sources, and measuring a cell's metabolic activity in response to individual energy substrates present as a medium additive, information regarding metabolic pathway activity and sensitivity to chemicals can be elucidated. Differential responses to test molecules can be observed depending on available nutritional sources and the cellular genetic background. Exposure time of cells to each nutritional environment can also affect sensitivity. Through the incorporation of concomitant microscopic imaging, visual assessment of cellular health and morphology following adaptation to the individual carbon sources can be carried out in a label-free manner, leading to a more complete interpretation of each data set.

Here we demonstrate a method to combine cellular metabolic Phenotype MicroArrays™ and automated, digital widefield phase contrast microscopy to screen differential nutritional pathway usage of test cells, in addition to potential pathway interruption by an apoptotic compound. Maintenance of a 5% CO_2-95% air atmosphere at 37 °C in the Cytation™ 5 plate chamber ensured sample integrity and enabled measurements of both cell morphology by image analysis and tetrazolium dye reduction rates, a reporter of cell energy production and cell viability in different nutritional environments. Gen5™ Software permitted multiplexed dye reduction kinetic analysis, in addition to apoptotic cells per well calculations. Results confirmed the validity of the combined methods to deliver a better understanding of the metabolic and toxic effects of pro-apoptotic molecules on target cell types.

Materials and Methods

Materials

Cells

MDA-MB-231 RFP cells (Cat. No. AKR-251) were purchased from Cell Biolabs, Inc. (San Diego, CA). Cells were propagated in Advanced DMEM Medium (Cat. No. 12491-015) containing 10% Fetal Bovine Serum (Cat. No. 10437-028) and 1x Pen-Strep-Glutamine (Cat. No. 10378-016), from Life Technologies (Carlsbad, CA).

Cytation 5 Cell Imaging Multi-Mode Reader

Cytation 5 is a modular multi-mode microplate reader that combines automated digital microscopy and microplate detection. Cytation 5 includes filter- and monochromator-based microplate reading; the microscopy module provides up to 60x magnification in fluorescence, brightfield, color brightfield and phase contrast. With special emphasis on live-cell assays, Cytation 5 has incubation to 65 °C, shaking, CO_2/O_2 gas control and dual injectors for kinetic assays. The instrument was used to measure the absorbance of microplate cultures, and to capture images of cells for morphological analysis. Cell counting was done using the Gen5 Data Analysis Software.

Gen5 Data Analysis Software

Gen5 software controls the operation of the Cytation 5 for both automated digital microscopy and PMT-based microplate reading. Image analysis and subpopulation calculations allow for counting of apoptotic cells meeting pre-determined signal threshold and circularity requirements.

Phenotype MicroArrays™ PM-M TOX1 MicroPlate™

	1	2	3	4	5	6	7	8	9	10	11	12
A	α-D-Glucose	α-D-Glucose	α-D-Glucose	α-D-Glucose	α-D-Glucose	α-D-Glucose	α-D-Glucose	α-D-Glucose	α-D-Glucose	α-D-Glucose	α-D-Glucose	α-D-Glucose
B	Inosine	Inosine	Inosine	Inosine	Inosine	Inosine	Inosine	Inosine	Inosine	Inosine	Inosine	Inosine
C	D-Galactose	D-Galactose	D-Galactose	D-Galactose	D-Galactose	D-Galactose	D-Galactose	D-Galactose	D-Galactose	D-Galactose	D-Galactose	D-Galactose
D	D-Glucose-1-Phosphate	D-Glucose-1-Phosphate	D-Glucose-1-Phosphate	D-Glucose-1-Phosphate	D-Glucose-1-Phosphate	D-Glucose-1-Phosphate	D-Glucose-1-Phosphate	D-Glucose-1-Phosphate	D-Glucose-1-Phosphate	D-Glucose-1-Phosphate	D-Glucose-1-Phosphate	D-Glucose-1-Phosphate
E	Xylitol	Xylitol	Xylitol	Xylitol	Xylitol	Xylitol	Xylitol	Xylitol	Xylitol	Xylitol	Xylitol	Xylitol
F	α-Keto-Glutaric Acid	α-Keto-Glutaric Acid	α-Keto-Glutaric Acid	α-Keto-Glutaric Acid	α-Keto-Glutaric Acid	α-Keto-Glutaric Acid	α-Keto-Glutaric Acid	α-Keto-Glutaric Acid	α-Keto-Glutaric Acid	α-Keto-Glutaric Acid	α-Keto-Glutaric Acid	α-Keto-Glutaric Acid
G	D,L-β-Hydroxy-Butyric Acid	D,L-β-Hydroxy-Butyric Acid	D,L-β-Hydroxy-Butyric Acid	D,L-β-Hydroxy-Butyric Acid	D,L-β-Hydroxy-Butyric Acid	D,L-β-Hydroxy-Butyric Acid	D,L-β-Hydroxy-Butyric Acid	D,L-β-Hydroxy-Butyric Acid	D,L-β-Hydroxy-Butyric Acid	D,L-β-Hydroxy-Butyric Acid	D,L-β-Hydroxy-Butyric Acid	D,L-β-Hydroxy-Butyric Acid
H	Pyruvic Acid	Pyruvic Acid	Pyruvic Acid	Pyruvic Acid	Pyruvic Acid	Pyruvic Acid	Pyruvic Acid	Pyruvic Acid	Pyruvic Acid	Pyruvic Acid	Pyruvic Acid	Pyruvic Acid

Figure 1. PM-M TOX1 Plate Energetic Substrate Array.

The PM-M TOX1 MicroPlate™ (Biolog, Inc., Hayward, CA) is designed for use as a cell-based assay to examine dose response effects of chemicals that inhibit or stimulate metabolism and energy production in target cells. Eight substrates in the plate probe different metabolic pathways that may be used by animal cells to generate energy. One or more cellular pathways, employing different enzymes, are available for the metabolism of each substrate.

The 96-well microplate has eight rows, each of which is coated with a different carbon source that can be differentially metabolized by most mammalian cells to produce energy. These include α-D-Glucose, Inosine, D-Galactose, D-Glucose-1-Phosphate, Xylitol, α-Keto-Glutaric Acid, D,L-β-Hydroxy-Butyric Acid, and Pyruvic Acid (See Fig. 1). A proprietary tetrazolium-based dye chemistry is used with the Phenotype MicroArray plates. The colorimetric readout of the assays indicates the effect of the diverse metabolic substrates on supplying energy and cell components for specific cell lines. As such, they can be used to measure baseline energetics, as well as dose-related chemical inhibition or stimulation of specific energy metabolism pathways in cells.

Methods

Cell Preparation and Dispensing into Microplates

Prior to dispensing, cells were removed from the tissue culture flask by trypsin, and suspended in glucose-free IF-M1 medium (Biolog, Cat. No. 72301) supplemented with 5% Fetal Calf Serum, 0.3 mM Glutamine and 1X Pen-Strep at a concentration of $2.5x10^5$ cells/mL. MDA-MB-231 cells were dispensed in all wells of three PM-M TOX1 plates (Biolog, Cat. No. 14101) at 10,000 cells/well in 40 µL. The plates were then incubated at 37 °C under 5% CO_2 -95% air for 16 hours to allow for attachment.

Compound Preparation and Addition

Following the initial incubation period, a six-point titration series of the pro-apoptotic anti-tumor agent oridonin (Sigma-Aldrich; Cat. No.O9639) was prepared at 5-fold higher final concentrations so that when 10 µL of the series was added to the 40 µL medium in each of the three plates, final oridonin concentrations of 100, 10, 1, 0.1, 0.01, and 0 µM, in duplicate wells, were created (Fig. 2).

	1	2	3	4	5	6	7	8	9	10	11	12
A	100 µM	100 µM	10 µM	10 µM	1 µM	1 µM	0.1 µM	0.1 µM	0.01 µM	0.01 µM	0 µM	0 µM
B	100 µM	100 µM	10 µM	10 µM	1 µM	1 µM	0.1 µM	0.1 µM	0.01 µM	0.01 µM	0 µM	0 µM
C	100 µM	100 µM	10 µM	10 µM	1 µM	1 µM	0.1 µM	0.1 µM	0.01 µM	0.01 µM	0 µM	0 µM
D	100 µM	100 µM	10 µM	10 µM	1 µM	1 µM	0.1 µM	0.1 µM	0.01 µM	0.01 µM	0 µM	0 µM
E	100 µM	100 µM	10 µM	10 µM	1 µM	1 µM	0.1 µM	0.1 µM	0.01 µM	0.01 µM	0 µM	0 µM
F	100 µM	100 µM	10 µM	10 µM	1 µM	1 µM	0.1 µM	0.1 µM	0.01 µM	0.01 µM	0 µM	0 µM
G	100 µM	100 µM	10 µM	10 µM	1 µM	1 µM	0.1 µM	0.1 µM	0.01 µM	0.01 µM	0 µM	0 µM
H	100 µM	100 µM	10 µM	10 µM	1 µM	1 µM	0.1 µM	0.1 µM	0.01 µM	0.01 µM	0 µM	0 µM

Figure 2. Diagram of Oridonin Treatment.

Image-Based Monitoring of Cell Health and Kinetic Determination of Cellular Energetics

At 0, 24 and 48 hours post oridonin addition, one PM-M TOX1 plate was placed into the Cytation 5, previously set to 37 °C / 5% CO_2 atmosphere, to collect phase contrast images of each well using a 4x and 20x objective. After imaging, 10 µL Biolog Redox Dye Mix MB (6X) [Cat. No. 74352] containing 30 mM Glucose was added to each well and the plate immediately returned to the Cytation 5, where absorbance measurements at 590 and 750 nm were recorded approximately every 1.5 minutes over a 2 hour period.

Gen5 Quantification of Cell Health and Apoptotic Induction

Cellular analysis was performed using Gen5 software on the 4x phase contrast images captured. The number of total cells per image was counted morphologically through changes in contrast. Apoptotic cells were further distinguished via sub-population analysis, taking into account the increase in circularity and lower phase contrast signal exhibited by this cell type. Tables 1 and 2 describe the parameters used to count total and apoptotic cells with the phase contrast channel.

Phase Contrast Total Cell Analysis Parameters	
Threshold	6,000 RFU
Min. Object Size	10 µm
Max. Object Size	40 µm
Bright Objects on a dark background	Unchecked
Split touching objects	Checked
Advanced Options	
Evaluate Background On	5% of Lowest Pixels
Image Smoothing Strength	0
Background Flattening Size	Auto

Table 1. 4x Phase contrast image cellular analysis parameters for identifying total cells.

Phase Contrast Apoptotic Cell Analysis Parameters	
Threshold	6,000 RFU
Min. Object Size	10 µm
Max. Object Size	70 µm
Bright Objects on a dark background	Unchecked
Split touching objects	Checked
Advanced Options	
Evaluate Background On	5% of Lowest Pixels
Image Smoothing Strength	0
Background Flattening Size	Auto
Sub-Population Analysis	
Circularity	>0.4
Mean Phase Contrast Signal	<40,000

Table 2. 4x Phase contrast image cellular analysis parameters for identifying apoptotic cells.

Calculation of Redox Dye Reduction Rate

Rate of dye reduction was calculated from A_{590}-A_{750} values measured from microplate wells every 1.5 min by the Cytation 5. Plots of A_{590}-A_{750} values versus time were used to identify the earliest contiguous A_{590}-A_{750} values that were linear with time of incubation. Least squares were used to fit lines to these values and the results slopes were taken as the initial rate of dye reduction.

Results and Discussion

MDA-MB-231 Cell Metabolic Analysis

Energetic Pathway Utilization

To fully understand the effect of nutrients on a target cell type, it is beneficial to first ascertain the extent to which each is metabolized under extended incubation conditions. The usage of the eight diverse nutritional sources by the MDA-MB-231 cells was determined by measuring initial rates of dye reduction (performed after microscopy). These measurements were performed in the absence of apoptotic agent and at different incubation times.

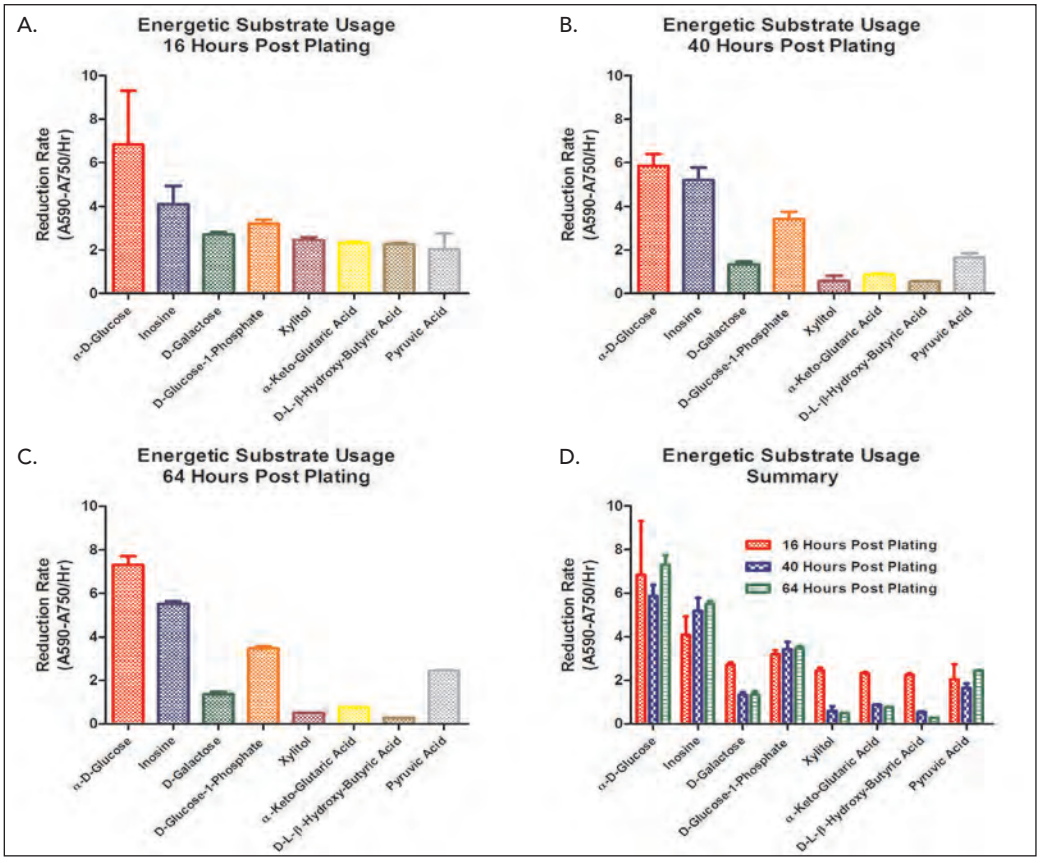


Figure 3. **MDA-MB-231 Energetic Pathway Utilization.** Initial rates of dye reduction calculated from cell cultures seeded at 10,000 cells/well in glucose-free IF-M1-based medium containing an indicated additive from the PM-M TOX1 plate and incubated for **(A)** 16, **(B)** 40 and **(C)** 64 hours. **(D)** Summary of all rates of dye reduction.

Following overnight cell incubation in the PM-M TOX1 plate, similar and moderate dye reduction rates were observed, with the exception of glucose supplemented cells which displayed higher reduction rates (Fig. 3A). After an additional 24 and 48 h of incubation in the medium with limiting nutritional sources (Figs. 3B and 3C), cells appear to maintain higher dye reduction rates when glucose, inosine, glucose-1-phosphate, and pyruvic acid were medium additives. The constant rates of dye reduction at each incubation time indicate preferential metabolism of these four additives compared to the other additives in PM-M TOX1, a consideration when examining toxic chemical challenge on energetic pathways.

Cell Health

Examination of cellular nutrition usage on overall cell health is also essential before proceeding with chemical screening. Test molecules may yield a greater potency in the presence of a particular nutrient. However, this potency may not be from the molecule itself, but rather from a general lack of viability caused by the inability of the cell to metabolize an additive. Incorporation of a label-free, live cell, image-based analysis can help make a proper assessment of the effects of nutritional source utilization.

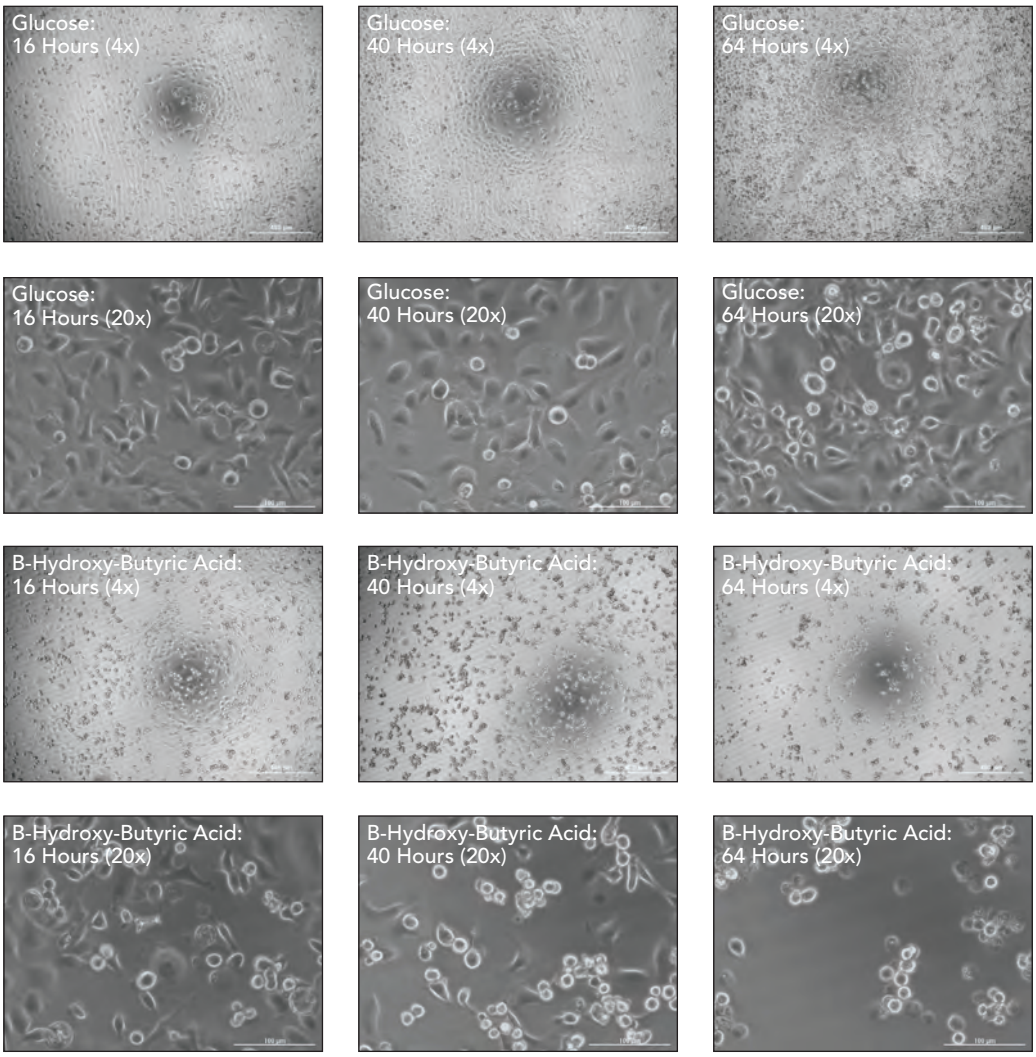
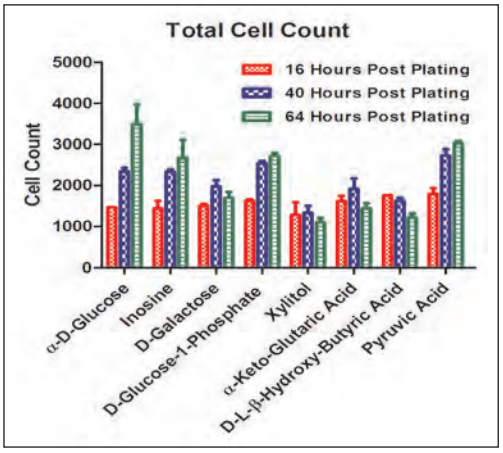


Figure 4. **Phase Contrast Imaging of MDA-MB-231 Cells.** Phase contrast images of MDA-MB-231 cells incubated in wells containing α-D-glucose or D,L-β-Hydroxy-Butyric Acid nutritional sources. Images captured using 4x or 20x magnification after 16, 40, and 64 hour incubation periods.

A.



B.

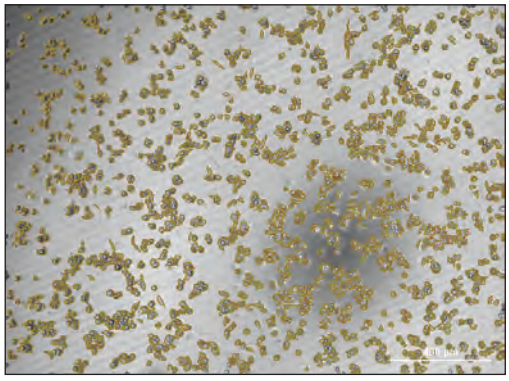


Figure 5. **Cell Count Determination.** **(A)** Total cells counted per image from nontreated wells containing unique nutritional sources. Phase contrast images captured using a 4x objective. **(B)** Cell analysis performed via Gen5 Data Analysis Software using the following primary analysis parameters (Yellow Masks): Threshold: 6000 RFU; Min. Object Size: 10 µm; Max. Object Size: 70 µm.

Cell health was qualitatively determined by inspecting 4x or 20x phase contrast images (Fig. 4). A larger number of total cells, as well as fully attached viable cells, were imaged from wells containing glucose as the medium supplement. The opposite was true with medium containing D,L-β-Hydroxy-Butyric Acid. A larger number of cells exhibiting classical morphologies of apoptosis, including cell rounding due to shrinkage and cytoplasm condensation[3], were observed as soon as 16 hours post plating. These morphology changes continued for an additional 24 h, before cell loss due to cellular necrosis and detachment from the bottom of the well was visualized after 64 hours of incubation.

These observations were also quantified using the 4x images and Gen5 Data Analysis Software, which identified and counted cellular objects within each image. It is apparent that cells maintained in wells whose nutrient (D-Galactose, Xylitol, α-Keto-Glutaric Acid, and D,L-β-Hydroxy-Butyric Acid) did not yield high dye reduction rates, exhibited low viability, became necrotic, and lost the ability to remain attached to the well bottom, rendering them undetected via cellular imaging (Fig. 5).

Compound Treatment Effect Determination

Impact of Energetic Substrate Metabolism

The effect of test molecules on differential nutritional metabolic pathways can also be examined. Oridonin, a natural tetracycline diterpenoid known to possess potent antitumor activity and induce apoptosis in a variety of cell types, was used as the model inhibitor. Differential substrate metabolism was once again assessed following multiple incubation periods with a titration of the small molecule.

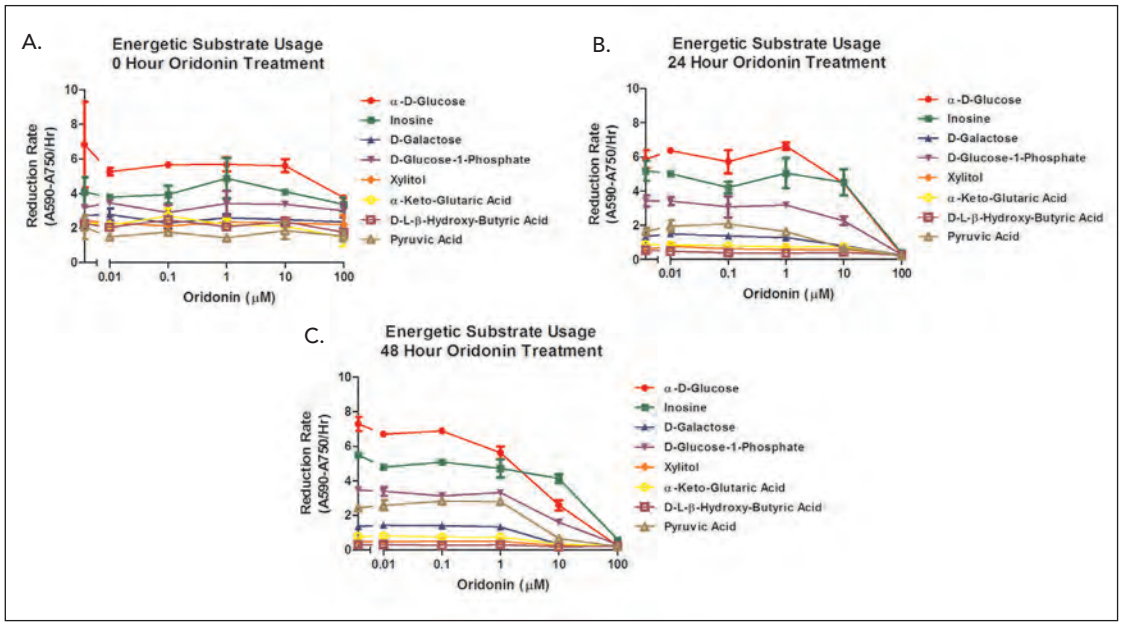


Figure 6. **Dye Reduction by cells following Oridonin Exposure.** Initial dye reduction rates calculated following a **(A)** 0, **(B)** 24 or **(C)** 48 h incubation of cells with oridonin concentrations ranging from 0-100 μM.

The results in Fig. 6 illustrate the time and dose dependent inhibitory effect of oridonin. Dye reduction rates quantified immediately after dosing show little to no fluctuation across all test concentrations. However, following a 24 h incubation period, dye reduction rates begin to decrease as drug concentrations increase. Except for cells with glucose supplementation, the potency of oridonin was similar when measured after a 48 h incubation in medium containing inosine, glucose-1-phosphate and pyruvate.

Total Cell Ramifications

The ability of oridonin to alter the integrity of cells appears to have a nutritional component. What is not known from this set of tests is the overall consequence on cell health while the cells are challenged with oridonin in different nutritive environments. Qualitative and quantitative image analysis of cells were performed prior to adding the redox dye to determine the whole cell phenotypic response to oridonin.

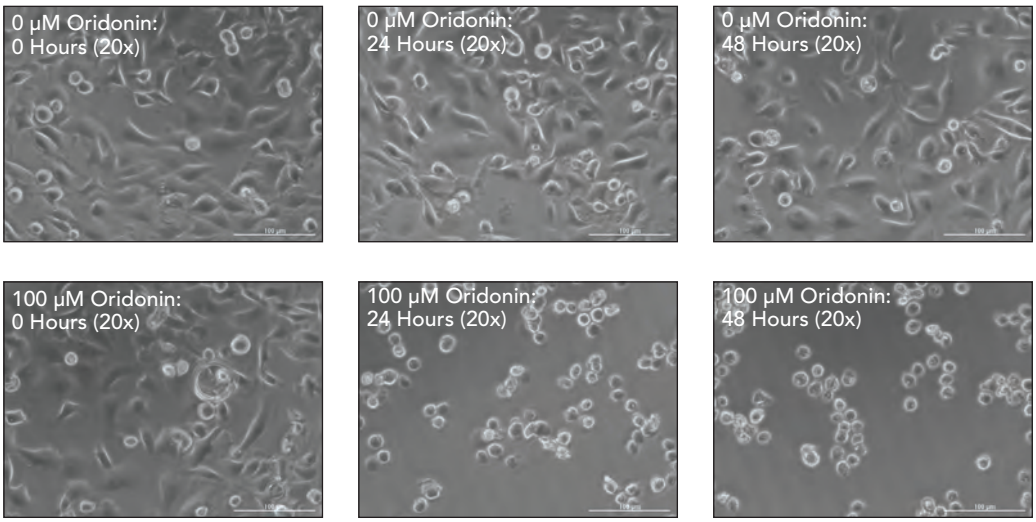


Figure 7. **Image-based Assessment of Oridonin Treatment.** Phase contrast images of MDA-MB-231 cells incubated in wells containing D-glucose-1-phosphate as a nutritional source, and treated with 0 or 100 µM oridonin. Images captured using 20x magnification after 0, 24 and 48 h incubation periods.

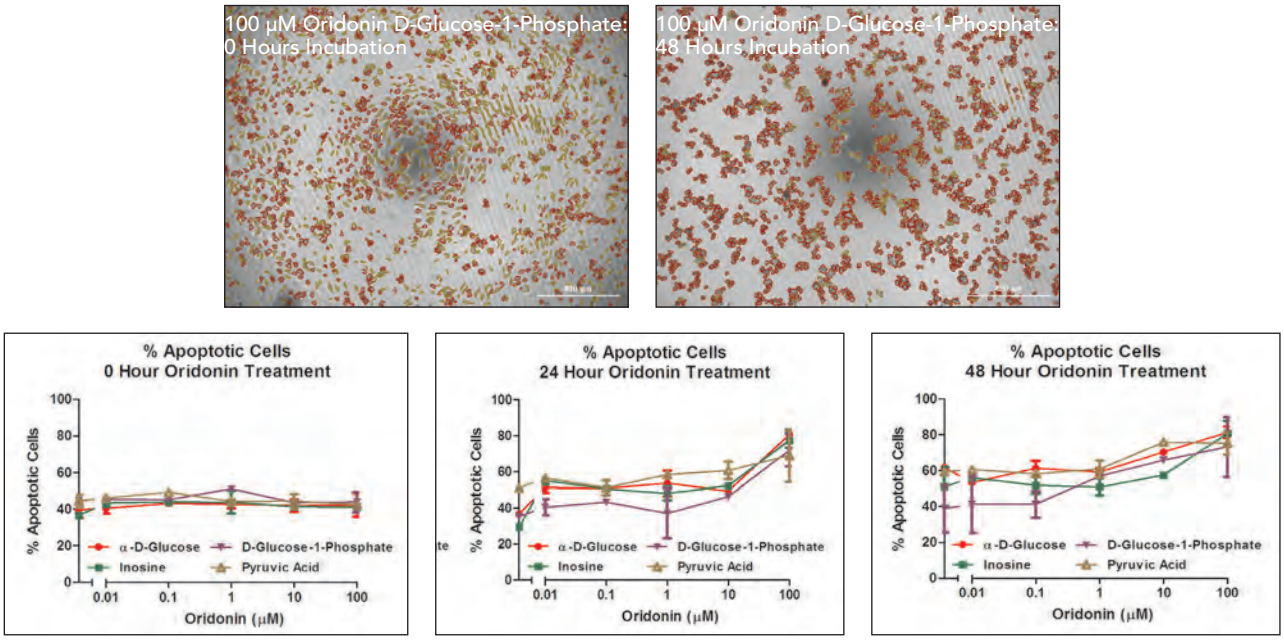


Figure 8. **Percent Apoptotic Cell Determination.** Total cell and apoptotic cell sub-population count performed per image. Phase contrast images captured using a 4x objective. Cell analysis performed via Gen5 Data Analysis Software. Total cell count primary analysis parameters (Yellow Masks): Threshold: 6000 RFU; Min. Object Size: 10 µm; Max. Object Size: 70 µm. Apoptotic cell sub-population analysis parameters (Red Masks): Circularity > 0.4; Mean Phase Contrast Signal: <40,000.

By performing a qualitative analysis of 20x images, higher numbers of apoptotic cells, as well as a decrease in total cell numbers, can be observed following oridonin treatment (Fig 7).

These findings can once again be substantiated quantitatively. Total cells were counted as previously explained in Fig. 5. Apoptotic cell numbers were then quantified via subpopulation analysis, by taking advantage of the circular morphology, and change in phase contrast signal exhibited by this cell type (Fig. 8A). The apoptotic/total cell ratio calculated for each additive validates qualitative findings, and confirms the cytotoxic effect which oridonin has on MDA-MB-231 cells[4].

Conclusions

Biolog's Phenotype MicroArrays™, in particular the PM-M TOX1 MicroPlate™, provide an easy-to-use, sensitive method to generate live cell multiplexed phenotypic analysis of differential energetic pathway utilization and dose response to chemical inhibition. When combined with the Cytation 5 a multiparametric analysis application can be performed. The colorimetric capabilities and environment control within the Cytation 5 allow kinetic analysis of dye reduction within varying nutritional environments. Incorporation of phase contrast imaging then provides additional phenotypic analysis on a whole cell level to evaluate the effects of nutrient usage, in addition to changes in morphology brought on by test molecule treatment. Finally, cellular analysis allows the determination of total and apoptotic cell numbers per image and provides quantitative assessment of phenotypes. The combination of Phenotype MicroArray technology and image-based analysis provide a multiplexed, label-free, live cell evaluation that can be used to accurately characterize target cell phenotypes and new potential lead molecules.

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